

**United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol**

**SAM 105**

**Supplemental Assay Method for the Titration of Infectious Bovine  
Rhinotracheitis Virus in Vaccines**

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**Supplemental Assay Method for the Titration of Infectious Bovine Rhinotracheitis Virus in Vaccines**

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**Supplemental Assay Method for the Titration of Infectious Bovine Rhinotracheitis Virus in Vaccines**

**1. Introduction**

This Supplemental Assay Method (SAM) describes an *in vitro* test method which utilizes viral plaque-forming units (PFU) in a cell culture system to titer infectious bovine rhinotracheitis virus (IBR) in modified-live veterinary vaccines.

**Note: For this SAM, the dilution terminology of 1:5 specifies 1 part plus 4 parts (liquid).**

**2. Materials**

**2.1 Equipment/instrumentation**

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

**2.1.1** Incubator,  $36^{\circ} \pm 2^{\circ}\text{C}$ ,  $5\% \pm 1\%$   $\text{CO}_2$ , high humidity (Model 3158, Forma Scientific Inc.)

**2.1.2** Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

**2.1.3** Blender

**2.1.4** Micropipettor, 200- $\mu\text{L}$ , 1000- $\mu\text{L}$ , and tips

**2.1.5** Water bath

**2.1.6** Self-refilling, repetitive syringe, 2-mL

**2.1.7** Media bottle, borosilicate glass with screw-top lid, 1000-mL

**2.2 Reagents/supplies**

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

**2.2.1** IBR Reference, Cooper strain (available from the Center for Veterinary Biologics [CVB])

**2.2.2** Madin-Darby bovine kidney (MDBK) or other susceptible cells found to be free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

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**2.2.3 Minimum essential medium (MEM) (National Veterinary Services Laboratories [NVSL] Media #20030)**

1. 9.61 g MEM with Earles salts without bicarbonate
2. 1.1 g sodium bicarbonate ( $\text{NaHCO}_3$ )
3. Dissolve **Sections 2.2.3(1)** and **2.2.3(2)** with 900 mL deionized water (DI).
4. Add 5 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to  $60^\circ \pm 2^\circ\text{C}$  until dissolved. Add to **Section 2.2.3(3)** with constant stirring.
5. Q.S. to 1000 mL with DI; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).
6. Sterilize through a 0.22- $\mu\text{m}$  filter.
7. Aseptically add:
  - a. 10 mL L-glutamine (200 mM)
  - b. 50  $\mu\text{g/mL}$  gentamicin sulfate
8. Store at  $2^\circ - 7^\circ\text{C}$ .

**2.2.4 Growth Medium**

1. 900 mL of MEM
2. Aseptically add 100 mL gamma-irradiated fetal bovine serum (FBS)
3. Store at  $2^\circ - 7^\circ\text{C}$ .

**2.2.5 2X Medium**

1. 19.22 g MEM with Earles salts without bicarbonate
2. 2.2 g  $\text{NaHCO}_3$
3. Dissolve with 900 mL DI.
4. Add 5 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to  $60^\circ \pm 2^\circ\text{C}$  until dissolved. Add to **Section 2.2.5(3)** with constant stirring.

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5. Q.S. to 1000 mL with DI and adjust to pH 6.8-6.9 with 2N HCl.
6. Sterilize through a 0.22- $\mu$ m filter.
7. Store at 2° - 7°C.

**2.2.6 2% Tragacanth Gum (Trag)**

1. 20 g Trag
2. 1000 mL DI
3. Using a blender, slowly add small amounts of Trag to 1 liter DI while vigorously mixing.
4. Sterilize by autoclaving at 15 psi, 121°  $\pm$  2°C for 35  $\pm$  5 minutes.
5. Pour 500 mL each into 1000-mL media bottles.
6. Store at 2° - 7°C.

**2.2.7 7.5% Sodium Bicarbonate**

1. 7.5 g NaHCO<sub>3</sub>
2. Q.S. to 100 mL with DI.
3. Sterilize through a 0.22- $\mu$ m filter.
4. Store at room temperature.

**2.2.8 70% Ethyl Alcohol**

1. 73 mL ethyl alcohol, denatured, 190 proof
2. 27 mL DI
3. Store at room temperature.

**2.2.9 Crystal Violet Stain**

1. 7.5 g crystal violet
2. 50 mL 70% ethyl alcohol

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3. Dissolve crystal violet in alcohol.
4. Add 250 mL formaldehyde.
5. Q.S. to 1000 mL with DI.
6. Filter through a Whatman #1 filter paper.
7. Store at room temperature.

**2.2.10** Tissue culture plates, 6-well

**2.2.11** Polystyrene tubes, 12 x 75-mm

**2.2.12** Polystyrene tubes, 17 x 100-mm

**2.2.13** Pipette, 25-mL and 10-mL

**2.2.14** Graduated cylinders, 25-, 50-, 100-, and 250-mL, sterile

**2.2.15** Parainfluenza-3 antiserum (PI3 AS) [available from CVB]

**2.2.16** Bovine viral diarrhea antiserum (BVD AS) [available from CVB]

**2.2.17** Bovine respiratory syncytial virus antiserum (BRSV AS) [available from CVB]

**3. Preparation for the test**

**3.1 Personnel qualifications/training**

Personnel must have training in virus titration assays, cell culture maintenance, and the principles of aseptic techniques.

**3.2 Preparation of equipment/instrumentation**

On the day of test initiation, set the water bath at  $36^{\circ} \pm 2^{\circ}\text{C}$ .

**3.3 Preparation of reagents/control procedures**

**3.3.1** MDBK Plates. Two days prior to test initiation, seed 1, 6-well tissue culture plate per Test Vaccine and 1, 6-well tissue culture plate for the control with MDBK cells, in Growth Medium, at a cell count that will produce a

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monolayer after 2 days  $\pm$  6 hours of incubation at  $36^{\circ}\pm 2^{\circ}\text{C}$ . These become the MDBK Plates.

**3.3.2** IBR Positive Control. On the day of test performance, rapidly thaw a vial of IBR Reference in a water bath at  $36^{\circ}\pm 2^{\circ}\text{C}$ . Tenfold dilutions are made as follows:

1. Add 4.5 mL of MEM into 6, 17 x 100-mm polystyrene tubes labeled  $10^{-1}$  to  $10^{-6}$  respectively.
2. Using a micropipettor, transfer 500  $\mu\text{L}$  of IBR Reference to the  $10^{-1}$  tube; mix by vortexing. Discard pipette tip.
3. Using a new pipette tip, transfer 500  $\mu\text{L}$  from the  $10^{-1}$ -labeled tube to the  $10^{-2}$  tube; mix by vortexing and discard tip.
4. Repeat **Section 3.3.2(3)** for each of the subsequent dilutions, transferring 500  $\mu\text{L}$  from the previous dilution to the next dilution tube until the dilution sequence is completed.

**3.3.3** Overlay Medium may be made ahead of testing or on day of test. Volumes listed are for 1 liter of overlay medium. Approximately 20 mL per plate is required.

1. Aseptically add to 500 mL of 2X Medium:
  - a. 50 mL gamma-irradiated FBS
  - b. 10 mL of 7.5% Sodium Bicarbonate
  - c. 50  $\mu\text{g/mL}$  gentamicin sulfate
  - d. 500 mL of 2% Trag
2. Mix and warm the Overlay Media in a  $36^{\circ}\pm 2^{\circ}\text{C}$  water bath for  $60 \pm 10$  minutes prior to performing the procedure in **Section 4.5**.

### 3.4 Preparation of the Test Vaccine

**3.4.1** The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). On the day of test initiation, remove the seal and stopper from both the Test Vaccine bottle and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the Test Vaccine's total volume indicated on the manufacturer's label (e.g., for a 50-dose container of 2 mL per dose, reconstitute with 100 mL of diluent). Aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing.

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**3.4.2**  $10^{-1}$  dilution of the Test Sample. Polyvalent vaccines require neutralization of the BVD, PI<sub>3</sub>, and/or BRSV fractions with antiserum prior to titering for IBR.

**1. IBR monovalent vaccine**

Mix 0.5 mL of the rehydrated Test Vaccine with 4.5 mL of Diluent Medium in a 17 x 100-mm polystyrene tube labeled  $10^{-1}$ ; mix by vortexing.

**2. IBR/PI<sub>3</sub> polyvalent vaccine**

**a.** Mix 1.0 mL of the rehydrated Test Vaccine with 4.0 mL of Diluent Medium in a 17 x 100-mm polystyrene tube labeled 1:5; mix by vortexing.

**b.** Mix 500  $\mu$ L from **Section 3.4.2(2.a)** with 500  $\mu$ L PI<sub>3</sub> AS in a 12 x 75-mm polystyrene tube labeled  $10^{-1}$ ; mix by vortexing.

**c.** Incubate at room temperature for  $60 \pm 15$  minutes.

**3. IBR/BVD/PI<sub>3</sub> polyvalent vaccine**

**a.** Mix 1.0 mL of the rehydrated Test Vaccine with 4.0 mL of Diluent Medium in a 17 x 100-mm polystyrene tube labeled 1:5; mix by vortexing.

**b.** Mix 1.0 mL from **Section 3.4.2(3.a)** with 500  $\mu$ L of BVD AS and 500  $\mu$ L of PI<sub>3</sub> AS in a 12 x 75-mm polystyrene tube labeled  $10^{-1}$ ; mix by vortexing.

**c.** Incubate at room temperature for  $60 \pm 15$  minutes.

**4. IBR/BVD/PI<sub>3</sub>/BRSV polyvalent vaccine**

**a.** Mix 2.0 mL of the rehydrated Test Vaccine with 8.0 mL of Diluent Medium in a 17 x 100-mm polystyrene tube labeled 1:5; mix by vortexing.

**b.** Mix 1.5 mL from **Section 3.4.2(4.a)** with 500  $\mu$ L of BVD AS, 500  $\mu$ L of PI<sub>3</sub> AS, and 500  $\mu$ L of BRSV AS in a 12 x 75-mm polystyrene tube labeled  $10^{-1}$ ; mix by vortexing.

**c.** Incubate at room temperature for  $60 \pm 15$  minutes.



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**3.4.3** Three tenfold dilutions are made from the  $10^{-1}$  dilution of the Test Vaccine using MEM.

- 1.** Pipette 4.5 mL of MEM into each of 3, 17 x 100-mm polystyrene tubes labeled  $10^{-2}$  through  $10^{-4}$  respectively.
- 2.** Pipette 500  $\mu$ L of the Test Vaccine from the  $10^{-1}$  tube into the  $10^{-2}$  tube; mix by vortexing. Discard pipette tip.
- 3.** Repeat **Section 3.4.3(2)** for each of the subsequent dilutions, transferring 500  $\mu$ L from the previous dilution to the next dilution tube until the dilution sequence is completed.

**4. Performance of the test**

- 4.1** On the day of test initiation, decant the Growth Media from the MDBK Plates.
- 4.2** Add 100  $\mu$ L/well from each dilution of the Test Vaccine to 2 wells of an MDBK Plate; mix by gentle swirling. Pipette tip changes are not necessary between each dilution in a series when pipetting from the most dilute to the most concentrated (e.g.,  $10^{-4}$  through  $10^{-2}$ ).
- 4.3** Add 100  $\mu$ L/well from each dilution of the IBR Positive Control to 2 wells of an MDBK Plate; mix by gentle swirling.
- 4.4** Maintain 2 wells as uninoculated cell culture controls.
- 4.5** Incubate inoculated MDBK Plates for virus adsorption at  $36^{\circ} \pm 2^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator for  $60 \pm 10$  minutes.
- 4.6** Add 3 mL/well of Overlay Medium (**Section 3.3.3**) to the MDBK Plates. Discard any unused, warmed Overlay Medium.
- 4.7** Incubate the MDBK Plates undisturbed at  $36^{\circ} \pm 2^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator for 4 days.
- 4.8** Following incubation, decant Overlay Medium. Pipette 2 mL of the Crystal Violet Stain (**Section 2.2.9**) into each well of an MDBK Plate, using the repetitive syringe.
- 4.9** Allow the MDBK Plates to incubate at room temperature for  $15 \pm 5$  minutes.

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**4.10** Wash the Crystal Violet Stain from the cell monolayers by dipping each MDBK Plate several times in a container of running cold tap water. Allow to air dry.

**4.11** The plaques are visible by naked eye as clear, circular areas in the cell monolayer where the cells have been destroyed by the virus.

**4.11.1** Count the number of plaques for each well. The plates should be read macroscopically.

**4.11.2** Average the number of plaques between the duplicate wells for each Test Vaccine and IBR Positive Control dilution.

**4.12** To determine the virus titer for each Test Vaccine and IBR Positive Control and express as PFU/dose, use the average from the dilution that contains between 10 and 100 PFU.

Example:

$\text{Log}_{10}$  of plaque count (30) 1.48

$\text{Log}_{10}$  of dilution counted ( $10^{-3}$ ) 3.00

$\text{Log}_{10}$  of 2-mL-dose factor

$\frac{\text{2-mL dose}}{100 \mu\text{l inoculum (20)}}$  1.30

Virus titer (total) 5.78

The Test Vaccine contains  $10^{5.78}$  PFU per dose.

**4.13** One plaque represents a single infective unit (IU), whereas 1, 50% tissue culture infective dose (TCID<sub>50</sub>) is statistically equivalent to a theoretical 0.69 IU. A TCID<sub>50</sub> endpoint will be 1.44 times those expressed as PFU per unit of inoculation. Therefore, to express PFU titer as TCID<sub>50</sub> titer, multiply the PFU titer by 1.44 or add 0.16 (the log of 1.44) to the log<sub>10</sub> value of the PFU titer. In the example above, the TCID<sub>50</sub> would be 5.78 + 0.16 = 5.94 or  $10^{5.94}$  TCID<sub>50</sub> per 2-mL-dose.

**5. Interpretation of the test results**

**5.1** For a valid test:

**5.1.1** No visible contamination shall be observed in all dilutions of a Test Vaccine.

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**5.1.2** The Test Vaccine and the IBR Positive Control must have dilutions in which 10 to 100 PFU are counted.

**5.1.3** The calculated titer of the IBR Positive Control must fall within plus or minus 2 standard deviations ( $\pm 2$  SD) of its mean titer, as established from a minimum of 10 previously determined titers.

**5.1.4** The Negative Cell Control shall be negative for plaques.

**5.1.5** If the validity requirements are not met, then the test is considered a **NO TEST** and may be retested without prejudice.

**5.2** If the titer of the Test Vaccine in a valid test is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Vaccine is **SATISFACTORY**.

**5.3** If the validity requirements are met but the titer of the Test Vaccine is less than the required minimum titer contained in the APHIS filed Outline of Production for the product under test, the Test Vaccine may be retested in accordance with the 9 CFR, Part 113.8.b.

**6. Report of test results**

Report test results as the virus titer in PFU per dose or TCID<sub>50</sub> per dose.

**7. References**

**7.1** Code of Federal Regulations, Title 9, Part 113.310, U.S. Government Printing Office, Washington, DC, 2007.

**7.2** Conrath TB. *Handbook of Microtiter Procedures*. Clinical and Research Applications Laboratory, Cooke Engineering Company, Alexandria, VA, 1972.

**7.3** Davis, Dulbecco, Eisen, and Ginsberg, ed., *Microbiology including Immunology and Molecular Genetics*, 3<sup>rd</sup> ed. Harper and Row, Hagerstown, MD, pg 880.

**8. Summary of revisions**

**Version .03**

- Kenneth Eernisse has been replaced by Joseph Hermann as a Contact.

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**Supplemental Assay Method for the Titration of Infectious Bovine Rhinotracheitis Virus in Vaccines**

- The phrase “Reference Control” has been changed to “Positive Control” throughout the document.

**Version .02**

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2.3** The formulation for the Diluent Medium has been changed and penicillin, streptomycin and amphotericin B have been removed.
- **4.7** The incubation time for plates prior to reading was changed from three to four days to facilitate reading of plaques.
- **4.11** The statement that the plates should be read macroscopically was added to clarify reading of plates.
- **4.12** The determination of virus titer for each Test Vaccine was rewritten to correct errors in calculations using the example given.
- The refrigeration temperatures have been changed from  $4^{\circ}\pm 2^{\circ}\text{C}$  to  $2^{\circ}-7^{\circ}\text{C}$ . This reflects the parameters established and monitored by the Rees system.
- “Test Serial” has been changed to “Test Vaccine” throughout the document.